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Supplemental Information

Inhibition of RNA Polymerase I as a Therapeutic Strategy to Promote Cancer-Specific Activation of p53

Megan J. Bywater, Gretchen Poortinga, Elaine Sanij, Nadine Hein, Abigail Peck, Carleen Cullinane, Meaghan Wall, Leonie Cluse, Denis Drygin, Kenna Anderes, Nanni Huser, Chris Proffitt, Joshua Bliesath, Mustapha Haddach Michael K. Schwaebe, David M. Ryckman, William G. Rice, Clemens Schmitt, Scott W. Lowe, Ricky W. Johnstone, Richard B. Pearson, Grant A. McArthur, and Ross D. Hannan

Inventory of Supplemental Information

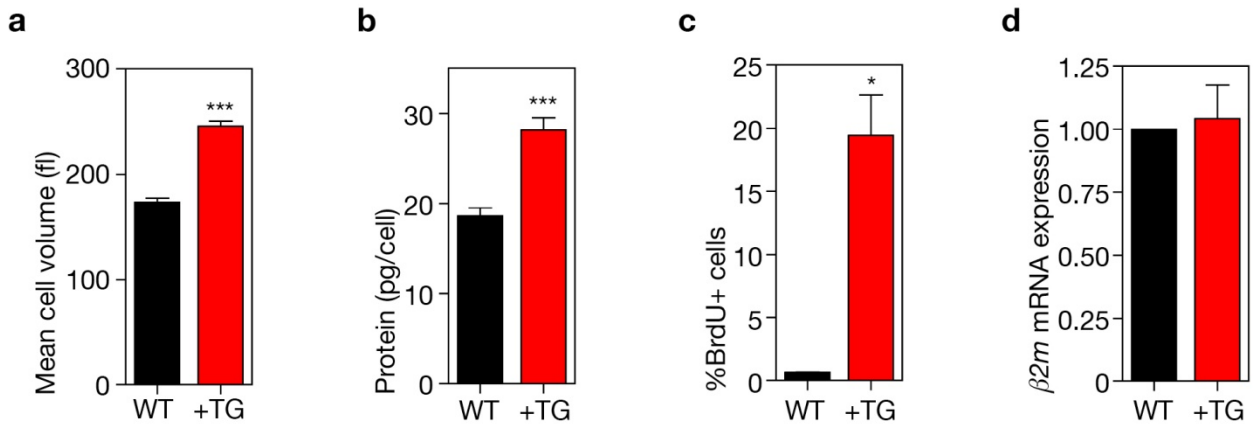

Figure S1, related to Figure 1**. Eμ-*Myc* premalignant B-cells exhibit increased rates of cell growth.** (**a**) Cell volume analysis ($p<0.0001$; $n=9$). (**b**) Total protein calculated per cell ($p<0.001$; $n=6$). (**c**) Percentage of BrdU positive cells ($p<0.05$; $n=3$). (**d**) Relative mRNA expression of *β2m*, determined by qRT-PCR ($p>0.05$; $n=5$). Eμ-*Myc* transgenic (+TG), wild type littermate (WT); Student's two-tailed t-test for all comparisons; Error bars represent SEM.

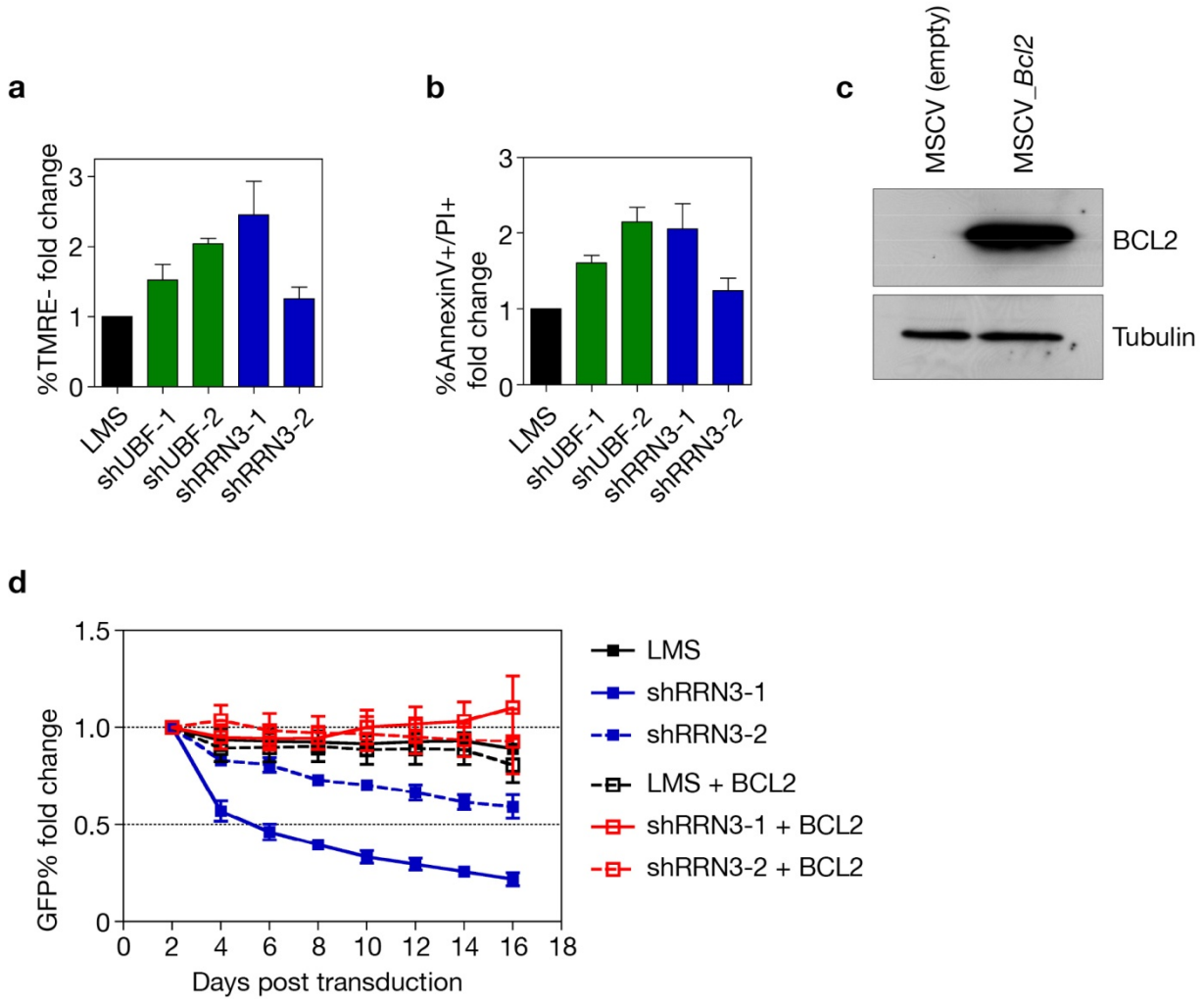

Figure S2, related to Figure 2**. Reducing pre-rRNA expression in Eμ-*Myc* lymphoma cells using shRNAmirs against *Ubf* or *Rrn3* strongly activates programmed cell death.**(**a**) Percentage of apoptotic cells determined by loss of TMRE staining, expressed as fold change over LMS (15%±2.5s.e.m.)(LMS vs shRRN3-1 $p<0.05$; shUBF $n=2$, shRRN3 $n=3$). (**b**) Percentage of apoptotic cell determined by Annexin V/PI co-staining, expressed as fold change over LMS (8.6%±1.8s.e.m.)(LMS vs shRRN3-1 $p<0.05$; shUBF $n=2$, shRRN3 $n=3$). (**c**) Western blot analysis of BCL2 overexpression. (**d**) GFP competition assay expressed as fold change in percentage of GFP+ cells compared to Day2 post transduction ($n=3$). Error bars represent SEM.

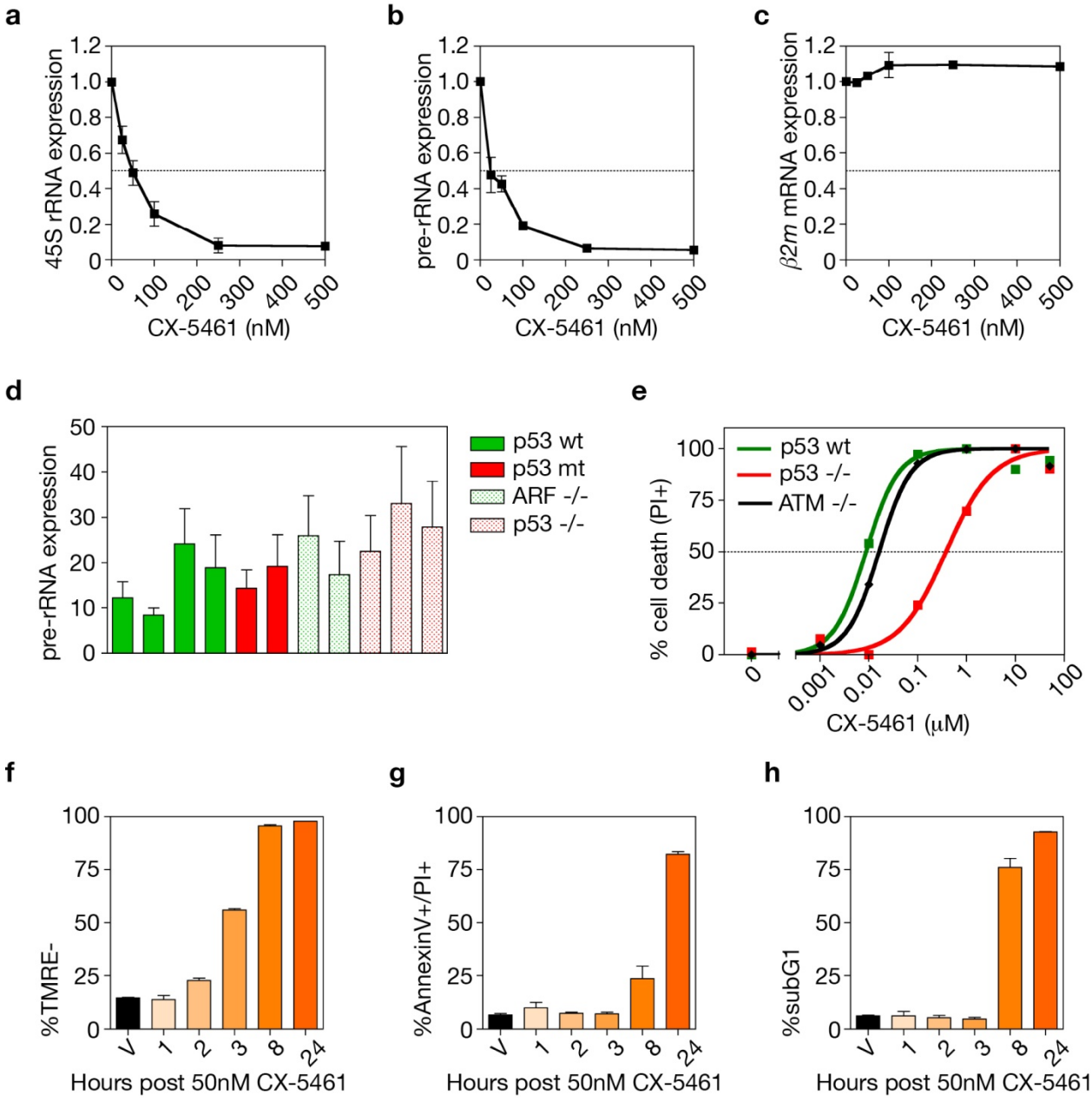

Figure S3, related to Figure 3**. CX-5461 induces rapid apoptotic cell death of p53 wild type Eμ-*Myc* lymphoma cells.** (**a**) Quantitation of 45S pre-rRNA synthesis as determined by [^{32}P] orthophosphate labeling (*n*=3). (**b**) Relative pre-rRNA expression determined by qRT-PCR (*n*=3). (**c**) Relative expression of *β2m* mRNA, determined by qRT-PCR (*n*=3). (**d**) Relative basal pre-rRNA expression in lymphoma cell lines (Figure 3C), as determined by qRT-PCR (*n*=3). (**e**) CX-5461 viability curve of Eμ-*Myc* lymphoma cell lines 16hrs post treatment, quantified by percentage of cells incorporating PI (representative of *n*=2). (**f**) Induction of apoptosis shown by loss of TMRE staining (*n*=1). (**g**) Induction of apoptosis as shown by increased Annexin V/PI co-staining (*n*=1). (**h**) Induction of apoptosis shown by an increasing number of cells with subG1 DNA content (*n*=1). Error bars represent SEM. (a-d); Error bars represent SD. (f-h).

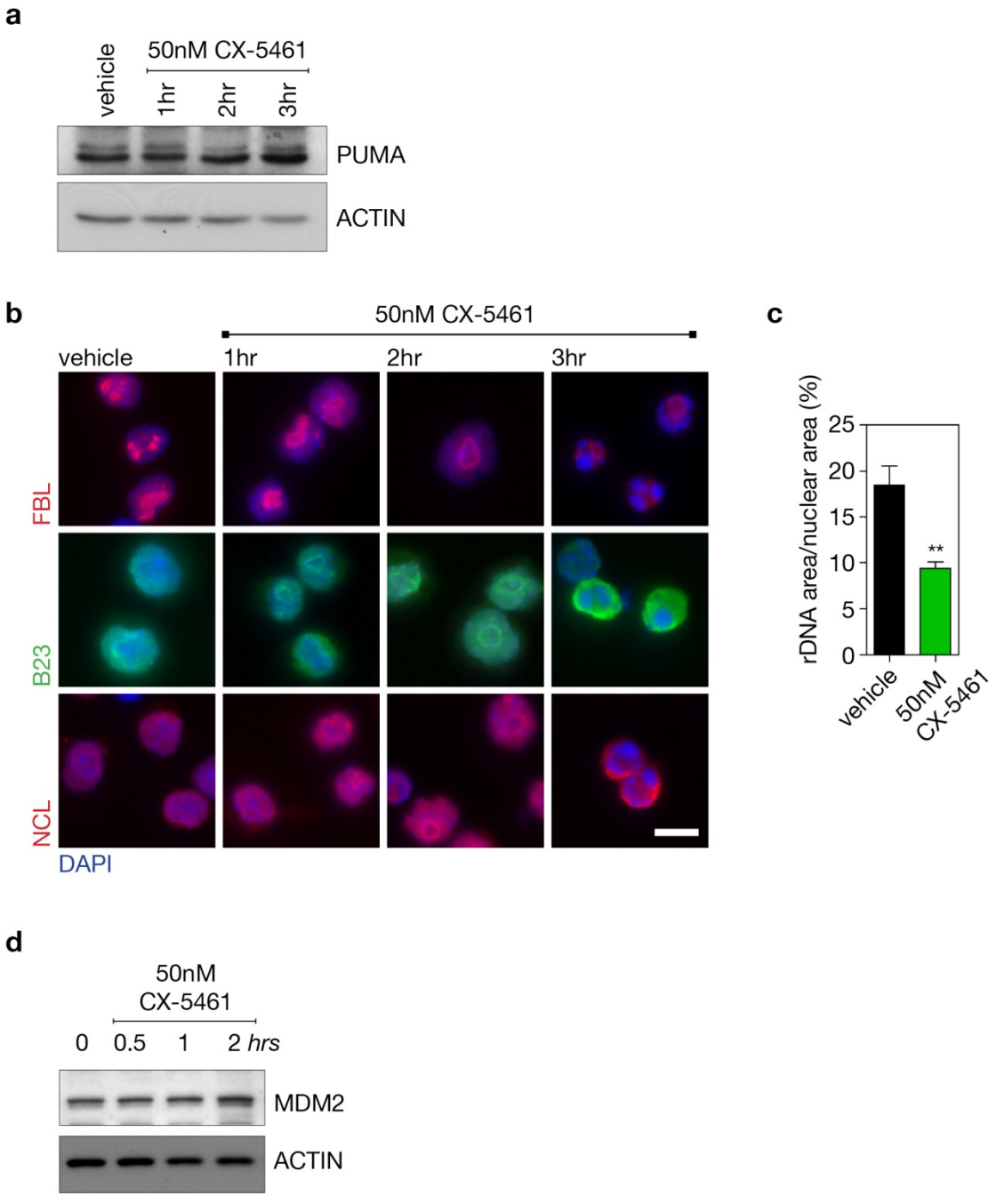

Figure S4, related to Figure 4**. CX-5461 induces rapid nucleolar disruption in p53 wild type Eμ-*Myc* lymphoma cells.** (**a**) Western blot analysis of Puma expression in p53 WT Eμ-*Myc* lymphoma cells in response to CX-5461 treatment. (**b**) Nucleolar disruption shown by Fibrillarin (FBL), Nucleophosmin (B23) and Nucleolin (NCL) immunofluorescence with DAPI counterstain, images taken at x60 magnification; scale bar, 10 μm. (**c**) Quantitation of FISH (Figure 4E), rDNA area expressed as a percentage of nuclear (DAPI) area; ($p<0.05$; $n=5$ fields). Student's two-tailed t-test; Error bars represent SEM. (**d**) Western blot analysis of MDM2 and ACTIN expression in p53 WT Eμ-*Myc* lymphoma cells in response to CX-5461 treatment.

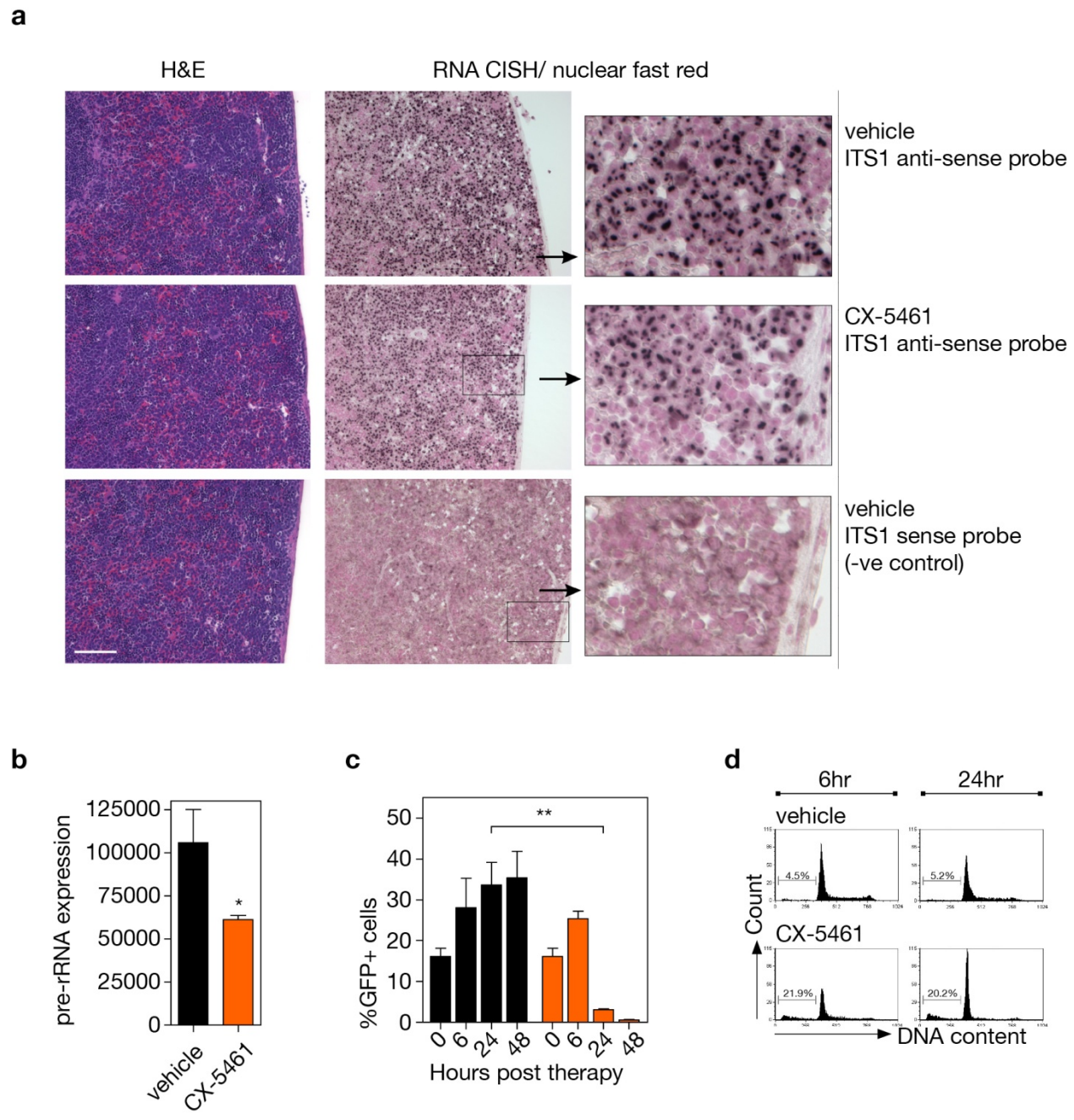

Figure S5, related to Figure 5**. Therapeutic administration of CX-5461 inhibits rDNA transcription and induces apoptosis of p53 wild type Eμ-*Myc* lymphoma cells in vivo.** (**a**) rRNA synthesis in the in Eμ-*Myc* lymphoma bearing spleens (Figure 5) at 1hr post treatment with 50mg/kg CX-5461, as determined by RNA chromogenic in situ hybridization (CISH) using an anti-sense probe to the ITS1; Images taken at x20 magnification; scale bar, 100 μm. (**b**) Quantitation of CISH (a) ($p<0.05$; $n=1$ spleen, 5 fields; average intensity). (**c**) Quantitation of FACS analysis of tumour burden in the lymph nodes (Figure 5B)(24hr vehicle vs 24hr CX5461 $p<0.01$; $n=3$). (**d**) Percentage of apoptotic cells in the lymph node, determined by subG1 DNA content analysis, quantitated in Figure 5E (representative of $n=3$). Student's two-tailed t-test (b), One-way ANOVA with Tukey's multiple comparison post test (c); Error bars represent SEM.

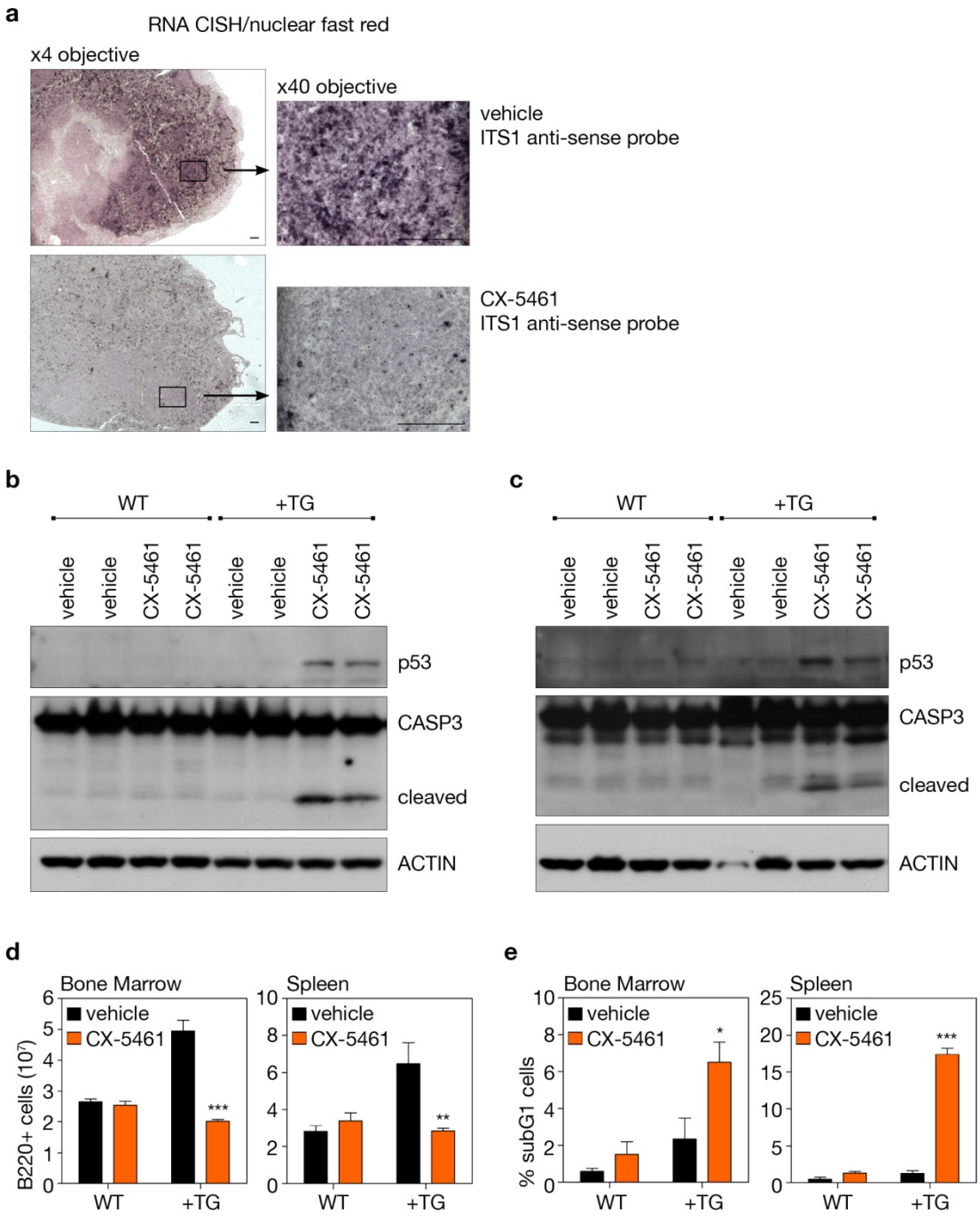

Figure S6, related to Figure 6**. Therapeutic administration of CX-5461 inhibits rDNA transcription in wild-type splenocytes but fails to activate p53 and induce apoptosis in vivo, in contrast to premalignant Eμ-*Myc* B-cells.** (**a**) rRNA synthesis in the spleen of wild-type mice at 1hr post treatment with 40mg/kg CX-5461, as determined by RNA chromogenic in situ hybridization (CISH) using an anti-sense probe to the ITS1; Images taken at x4 (left) and x40 (right, region highlighted in left image) magnification; scale bars, 100μm; Quantitation (Figure 6). (**b-c**) Western blot analysis of total p53 and CASPASE-3 cleavage in the bone marrow (a) and spleen (c) of 4-6 week Eμ-*Myc* transgenic mice and wild type littermates, 6hrs post treatment with vehicle or 40mg/kg CX-5461. (**d**) Number of B220+ cells in the bone marrow (2 femurs + 2 tibias) and spleen (half organ) at 6hrs post therapy (+TG, vehicle vs CX-5461; bone marrow $p<0.001$, spleen $p<0.01$; $n=4$). (**e**) Quantitation of apoptotic cells in the bone marrow and spleen of 4-6 week Eμ-*Myc* transgenic mice and wild type littermates, 6hrs post treatment with vehicle or 40mg/kg CX-5461; determined by subG1 DNA content analysis (+TG, vehicle vs CX-5461; bone marrow $p<0.001$, spleen $p<0.05$; n=4). Student's two-tailed t-test for all comparisons; Error bars represent SEM.

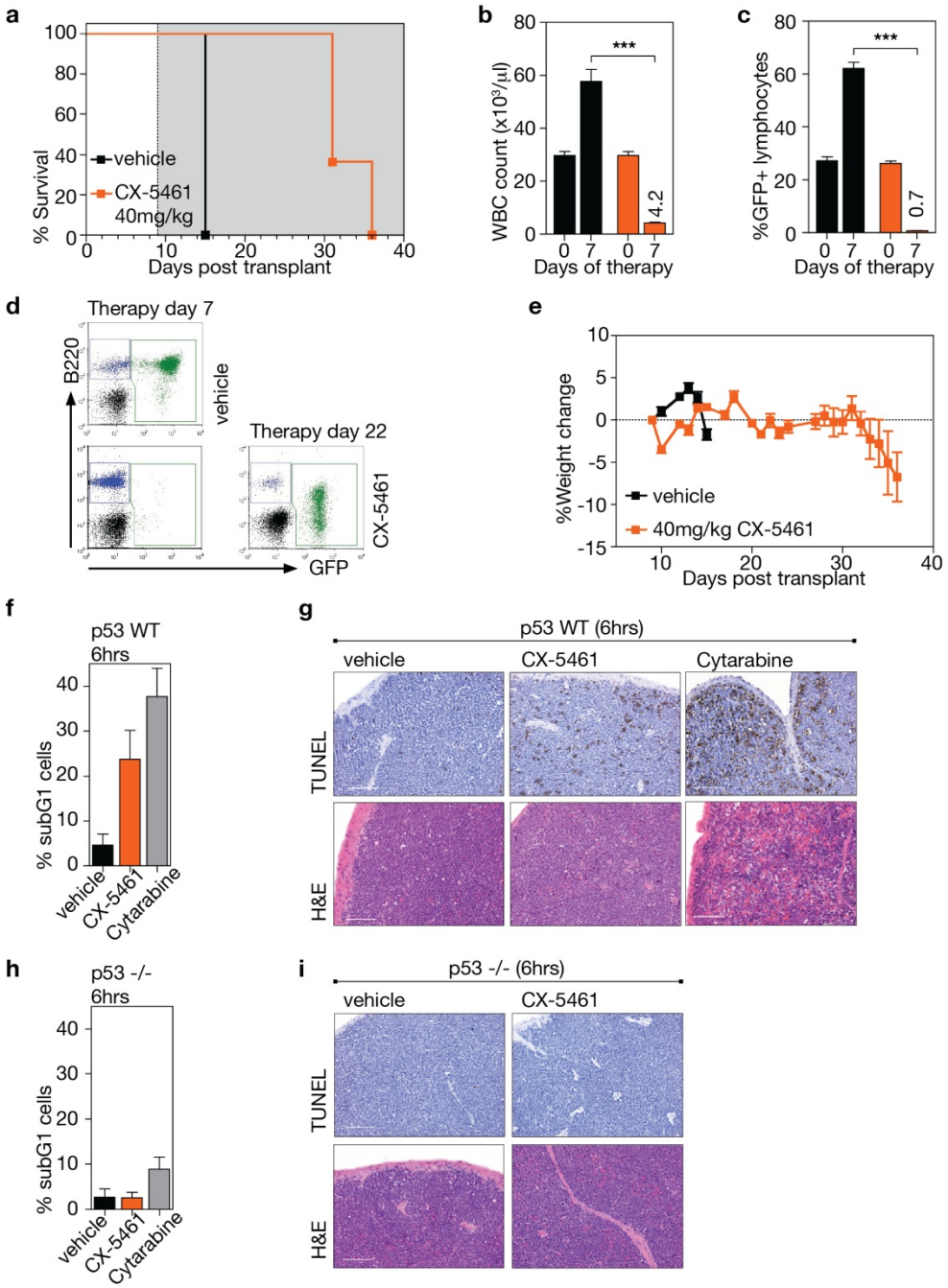

Figure S7, related to Figure 7**. Therapeutic administration of CX-5461 increases survival from transplanted p53 wild type 4242 Eμ-*Myc* lymphoma and selectively induces apoptotic cell death of p53 wild type AML1/ETO9a+Nras cells in vivo.** (**a**) Kaplan-Meier curves showing increased survival of mice transplanted with 4242 Eμ-*Myc* lymphoma and treated chronically with CX-5461 at 40mg/kg every 3 days in comparison to vehicle, commencing 9 days post transplant (period on therapy indicated by shaded area) ($p<0.0001$; $n=10$). (**b**) White blood cell count at 0 and 7 days post start of therapy (Day 7 vehicle vs Day 7 CX5461 $p<0.001$; $n=10$). (**c**) Quantitation of FACS analysis (d) of tumor burden in the peripheral blood at 7 days post start of therapy ($P<0.0001$; $n=10$). (**d**) Representative FACS analysis of tumor burden in peripheral blood at 7 and 22 days post start therapy. (**e**) Percent change in body weight of mice on therapy ($n=10$ at commencement of therapy). (**f&h**) Quantitation of apoptotic cells in spleen, determined by PI incorporation. FACS analysis at 6hrs (CX-5461 n=3, vehicle n=2, cytarabine n=3)) post treatment of 40mg/kg CX-5461. (**g&i**) Hematoxylin and Eosin (H&E) and TUNEL stained spleen sections showing leukemic infiltration and appearance of apoptotic cells in CX-5461 treated and cytarabine treated p53 WT AML, but not p53-/- cells. Images taken at x20 magnification; scale bar, 100 μm. Logrank test (a); One-way ANOVA with Tukey's multiple comparison post test (b-c); Error bars represent SEM (b&c); Error bars represent SD (f&h).

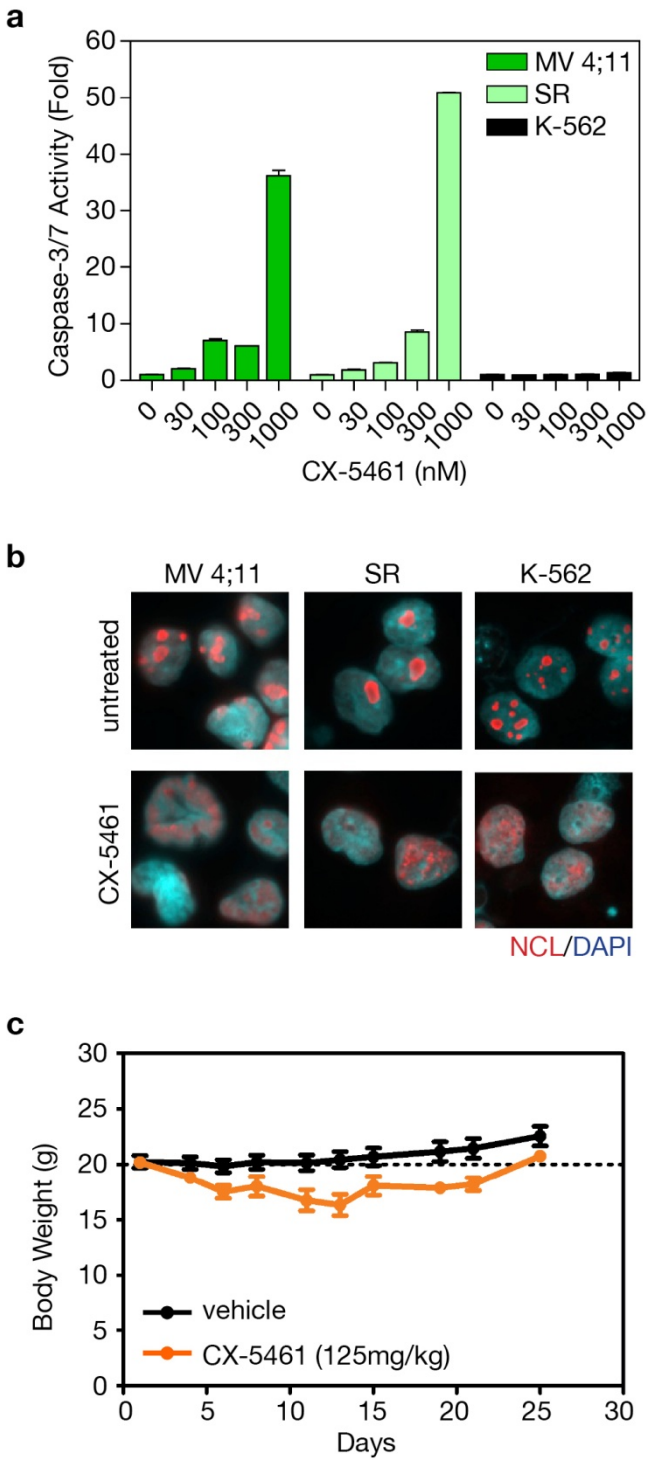

Figure S8, related to Figure 8**. Effect of CX-5461 in human leukaemia and lymphoma cell lines.** (**a**) CX-5461 induces apoptosis in p53 WT MV 4;11 and p53 WT SR, but not p53 null K562 cell lines. (**b**) CX-5461 disrupts nucleolar morphology in SR, MV 4;11 and K562 cells treated at 1μM for 4hrs, as shown by immunofluorescence for Nucleolin (NCL) with Dapi counterstain. (**c**) Effect of therapeutic treatment with CX-5461 on animal body weight in the MV 4;11 xenograft model (Figure 8E). Error bars represent SEM.

Supplemental Experimental Procedures

Short-hairpin RNA sequences cloned into the LMS expression construct.

	Sequence (5'-3')
shUBF-1	cgg aga tta tgc ggg act ata t
shUBF-2	ccc aga gaa gaa gaa gat gaa a
shRRN3-1	gga gtt taa gaa atc tac taa
shRRN3-2	gga cac tga ccc tga gaa gaa

RNA extraction and quantitative real-time PCR. An equal number of cells were lysed in Trizol reagent (Invitrogen) and RNA prepared using manufacturer's instructions. RNA was analyzed on an Agilent Bioanalyzer using the RNA 6000 Nano Assay kit according to the manufacturer's instructions. The relative percentage of 18S and 28S rRNA per sample was used to calculate the amount of rRNA per cell. cDNA was prepared and qRT-PCR performed and analyzed as described (Poortinga et al., 2004) with data normalized to *β2m* (*B2m)* mRNA levels. Primer sequences are previously published (Poortinga et al., 2004; Poortinga et al., 2011) or listed in below.

Target	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
45S rRNA ITS1	ccg gct tgc ccg att t	gcc agc agg aac gag
p21	aat acc gtg ggt gtc aaa gc	gtg tga gga ctc ggg aca at
Puma	ctg ggc act ggg tta aga ag	ggg ggt ctg tga aga gca ta
Odc1	gca tgc atc tgc ttg ata ttg g	gtg atc tct tca aat tta agc ttt gta tct
mTert	tgt gtt tcg gag aca tgg aga a	aag tca tca aca aaa cgt aaa agc a

Flow Cytometry. For competition assays, GFP+, LMS-shRNA-mir transduced cells sorted on a BD FACS Vantage SE were seeded along with equal numbers of mock transduced (retrovirus alone, no vector) and sorted GFP- cells. The percentage of GFP+ cells was analyzed on a BD FACS CantoII. Dead cells were detected by propidium iodine (PI) incorporation (0.01mg/ml, Sigma) and were excluded from analysis. To determine DNA content cells fixed in 95% ethanol were stained with PI (0.01mg/ml) and analyzed on a BD FACS CantoII. To determine apoptotic populations, live cells were incubated either with TMRE (100nM, Invitrogen) or APC conjugated Annexin V (BD Pharmingen, 550475) and PI (0.01mg/ml) and analyzed on a BD FACS CantoII. To determine tumor burden, either whole lymph node suspensions or whole blood (post red blood cell lysis) was stained with APC conjugated anti-B220

(CD45R) (eBioscience, RA3-6B2) and PI (0.01mg/ml) and analyzed on a BD FACS CantoII. All FACS data was analyzed using the FACSExpress Software. WBC counts were determined from peripheral whole blood on an Advia 120 hematology analyzer (Bayer).

Western blotting. Protein content per cell was determined using a D_C Protein Assay (Bio-Rad) with lysates generated from equal number of cells. 20-80 μg of whole cell lysate (50mM Tris pH6.8, 1% SDS, 10% glycerol, protease inhibitors) was resolved by SDS-PAGE, electrophoretically transferred onto PVDF membranes (Millipore) and analyzed using the anti-sera listed below and enhanced chemiluminescence (ECL) detection (GE Healthcare). For co-immunoprecipitation, 1 mg of cell lysate (50mM TrisHCl pH7.5, 150mM KCl, 5mM MgCl, 1mM EGTA, 10% glycerol, 0.8% NP40) sheared with a 25G needle was incubated with 3 μg of anti-MDM2 antibody, 1 μg each respectively of antibodies listed in below.

Protein	**Origin**	**Source**	**Clone/code**
Ubf	rabbit	in-house	n/a
c-Myc	rabbit	Cell Signalling	9402
Pol1rb	rabbit	in-house	n/a
Rrn3	rabbit	Brian McStay	n/a
Caspase3	mouse	BD Biosciences	611049
p53	mouse	Novacastra	NCL-p53-505
p53	rabbit	Vector Labs	CM5
p21	rabbit	Santa Cruz	C-19
Actin	mouse	MP Biomedicals	C4
Mdm2	mouse	Calbiochem	4B11
Mdm2	mouse	Calbiochem	2A10
Mdm2	mouse	Santa Cruz	SMP14
rpL5	rabbit	Sinisa Volarevic	G15.L5.4
rpL11	mouse	Invitrogen	3A4A7
Puma	rabbit	Cell Signalling	4976
Fibrillarin	rabbit	Abcam	ab5821
Nucleophosmin	mouse	Abcam	ab10530